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Extracellular urinary microRNAs as non-invasive biomarkers of endometrial and ovarian cancer

Antonija Hanžek (1), Christian Siatka (1) & Anne-Cécile E. Duc (1)

Abstract

Introduction

Gynecological cancers account for a large number of cancer-related deaths in women. Endometrial cancer is the most prevalent, while ovarian cancer is the deadliest gynecological cancer worldwide. To overcome the clinical need for easy and rapid testing, there is a growing interest in cancer detection in non-invasive modalities. With a growing field of liquid biopsy, urine became interesting source of cancer biomarkers.

Objectives

The aim of this manuscript is to provide an overview on the origin, analysis and the clinical significance of urine microRNAs in gynecological cancers, with a focus on ovarian and endometrial cancer. MicroRNAs, a class of small non-coding nucleic acids, are emerging as a non-invasive biomarkers due to the feasibility and the extreme stability in body fluids. Specific miRNA expression signatures have been previously identified in ovarian and endometrial cancer.

Results

The aim of this manuscript is to provide an overview on the origin, analysis and the clinical significance of urine microRNAs in gynecological cancers, with the focus on ovarian and endometrial cancer.

Conclusion

The advantages and limitations of urine microRNA utility and technologies are discussed. Previously detected microRNA from urine of the patients are summarized to evaluate their potential as non-invasive clinical biomarkers in gynecological oncology.

Keywords Ovarian cancer · Endometrial cancer · microRNA · Urine · Liquid biopsy · Biomarkers

Introduction

The tumors of the female genital tract represent a major cause of morbidity and mortality among women worldwide (Bray et al. [2018](#)). Endometrial cancer (EC) is the most prevalent, while ovarian cancer (OC) is the deadliest (Lortet-Tieulent et al. [2018](#)). When diagnosed in an early phase, 5-year survival is 81% for EC and 90% for OC (Berek et al. [2014](#); Makker et al. [2021](#)). However, survival rates drop drastically in late stages (advanced or metastatic disease) to 17% for EC (Makker et al. [2021](#)) and between 15 and 40% for OC (Berek et al. [2014](#)), respectively. Late diagnosis is a crucial factor contributing to poor prognosis and high mortality of these malignancies. Unfortunately, routine screenings that are sufficiently accurate and non-invasive are unavailable for either EC or OC (Braun et al. [2016](#); Menon et al. [2018](#)). However, unselected OC patients can carry detectable mutations in number of genes important for the clinical decision. Those pathogenic variations include genes that encode for proteins involved in DNA damage repair, such as BRCA1 and BRCA2, or genes involved in mismatch repair pathways, implicated in hereditary OC (Shah et al. [2022](#)). In addition, next-generation sequencing

revealed that up to a quarter of individuals with high-grade serous OC have mutations in homologous recombination effectors like PALB2, RAD51, ATM, BRIP1, BARD1, and CHEK2 (Shah et al. [2022](#)).

EC is diagnosed based on abnormal bleeding which is further clinically evaluated by physical examination, endometrial tissue sampling or ultrasonography (Braun et al. [2016](#)). Currently, there is no biomarker with adequate specificity and sensitivity for diagnosis or prognosis of EC (Hutt et al. [2019](#)). For OC, diagnosis relies on pelvic examination, transvaginal ultrasonography and increased serum level of cancer antigen 125 (CA125), which are not sensitive enough for early diagnosis (Ledermann et al. [2013](#)). Indeed, CA125 is found elevated in only 50% of patients with early stage and 85% of patients with advanced OC, limiting its utility in early diagnostics (Ledermann et al. [2013](#); Sölétormos et al. [2016](#)). Moreover, CA125 is not specific enough since it can also be elevated during menstruation and pregnancy, in benign gynecological conditions and other cancers, leading to false-positive results (Meden and Fattahi-Meibodi [1998](#); Sölétormos et al. [2016](#)). A second, more sensitive and specific OC biomarker is Human Epididymis protein 4 (HE4). The algorithm used in the differential diagnosis of pelvic masses, called Risk Of Malignancy Algorithm (ROMA), combines the serum levels of CA125 and HE4 with the menopausal status into a numerical score that predicts the risk for cancer (Moore et al. [2009](#)). Therefore, discovery of novel classes of biomarkers for these cancers is needed to advance early diagnosis and improve prognosis. Improvements in the various technologies, such as mass spectrometry (MS) and protein arrays for proteomics, or next-generation sequencing (NGS) for genomics analysis, have advanced the dissection of the underlying molecular events and characterization of the gynecological cancers and, therefore, pave the way for the discovery of new diagnostic and therapeutic targets (Ghose et al. [2022](#)).

In the last decades, the evolution of molecular biology methods to analyze small fragments of nucleic acids allowed to define new strategies for disease diagnosis. The small non-coding RNAs, called “microRNAs” or “miRNAs” are involved in the gene regulation. Recently, microRNAs emerged as a good candidates for cancer diagnosis. The stability and accessibility in body fluids make them particularly interesting as non-invasive biomarkers. These small non-coding RNAs, about 18–25 nucleotides in length, display aberrant expression in cancer, including both EC and OC (Delangle et al. [2019](#); Ghafouri-Fard et al. [2020](#)). Therefore, studying microRNAs levels could be part of a complementary approach to analysis of DNA (genome), mRNAs (transcriptome) and proteins (proteome), leading to the coining of the term miRNome. Confirmation of gynecological cancers requires invasive tissue biopsy. Since biopsy can be unpleasant for the patients, recent studies are focusing on alternative non-invasive sources of biomarkers, such as urine. With an ease of access and a proven stability in urine (Mall et al. [2013](#)), urinary miRNAs could contribute as biomarkers of early diagnosis, and help to overcome the need for novel diagnostic procedures in gynecological oncology.

In this review, we summarize miRNAs expression profiles in urine of EC and OC patients and highlight the potential candidate biomarkers. The urine miRNAs detection methodology is discussed with advantages and limitations in order to evaluate their potential to be used as non-invasive cancer biomarkers in clinical setting.

Biogenesis and function of miRNAs

Human genome encodes approximately 2600 mature miRNAs expressed from non-protein-coding genes located in the cancer-linked regions or fragile chromosomal sites, implying the roles in cancer (Saini et al. [2007](#); Plotnikova et al. [2019](#)). The biogenesis of the microRNAs is

presented in Fig. 1. In the dominant canonical pathway, miRNAs are transcribed by RNA polymerase II (Lee et al. 2004) or III (Borchert et al. 2006) into stem-loop structured *pri-miRNAs*, which are cleaved by a complex consisting of RNA-binding protein DGCR8 (DiGeorge Syndrome Critical Region 8) and the ribonuclease III enzyme Drosha into 60–70 bp hairpin miRNAs precursors, or *pre-miRNAs* (Winter et al. 2009). Once pre-miRNAs are generated, they are exported to the cytoplasm by an exportin 5 (XPO5)/RanGTP transport system (MacFarlane and Murphy 2010). The maturation in cytoplasm is mediated by the multi-protein complex composed of the RNase Dicer and associated proteins TRBP (Tar RNA binding protein), PACT (protein activator of PKR) and the core component Argonaute-2 (AGO2).

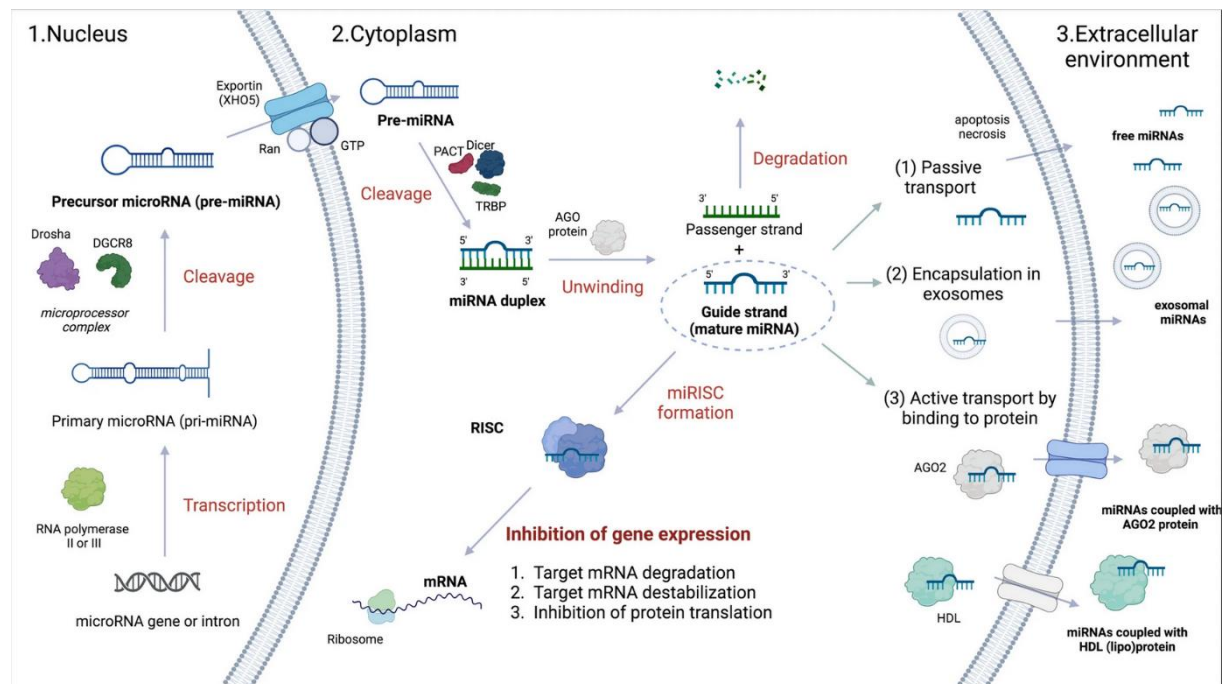


Fig. 1 Biogenesis pathway and the origin of the extracellular microRNAs. The miRNAs are transcribed into long primary miRNAs (*pri-miRNAs*) which are cleaved by microprocessor complex Drosha/DGCR8 into precursor miRNA (*pre-miRNA*). Pre-miRNA is transported into cytoplasm through Exportin (XHO5)/Ran GTP complex and cleaved by Dicer enzyme and associated PACT/TRBP proteins into miRNA duplex. MiRNA duplex is incorporated into an Argonaut (AGO) protein forming RNA-induced silencing complex (RISC). The duplex is unwound into two-single stranded miRNAs: the functional *guide strand* and the *passenger strand* which is degraded. The mature miRNA, as the part of the RISC, binds to 3' UTR region of target mRNAs and repress the gene expression by either mRNA degradation, mRNA destabilization or inhibition of translation. MiRNAs can be released to extracellular environment by (1) the passive leakage through cell death, (2) active encapsulation in exosomes or (3) bounding to AGO2 or HDL proteins. (Created with BioRender.com)

Dicer cleaves the pre-miRNA resulting in a double-stranded *miRNA duplex* approximately 21–23 nucleotides long. The miRNAs duplex then couples with AGO2 protein and together they form an RNA-induced silencing complex called RISC (Winter et al. 2009; O'Brien et al. 2018). The RNA duplex is separated by helicase into one functional strand, called *guide strand*, which is complementary to the target mRNA, and the *passenger strand*, which is degraded (O'Brien et al. 2018). In the nomenclature of the miRNAs, 5p/3p strand annotation indicate the position of the strand in the pre-miRNA hairpin (Desvignes et al. 2015). One of the strands remains

linked to AGO (mature miRNA) and plays a key role in the regulation of genes at the post-transcriptional level by targeting the mRNAs by binding of the “seed” region (nucleotides at position 2–8).

Once the functional RISC complex is formed, the degree of complementarity between RISC and target mRNA will determine which gene silencing effect occurs—full complementarity results in mRNA degradation, while partial complementarity causes destabilization or inhibition of translation (O’Brien et al. [2018](#)). Target mRNA is destabilized by deadenylation and decapping leading to its degradation. Translation of targeted mRNAs is also repressed by inhibiting the assembly of the preinitiation complex (O’Brien et al. [2018](#)).

MiRNAs have a wide spectrum of gene regulation activities. For example, the first identified and one of the most studied miRNAs in humans, the let-7 family (12 members), are involved in aging, stem cell differentiation, cell adhesion, proliferation and muscle formation. Indeed, let-7 is negatively regulated by the RNA-binding protein LIN-28 homolog A (LIN28), which controls the pluripotency of embryonic stem cells (Cheung et al. [2022](#)). Interestingly, it has been demonstrated that let-7 miRNA levels are decreased in LIN28-positive ovarian germ cell tumors, indicating that the LIN28/let-7 pathway may play a key role in the development of this histological subtype of non-epithelial OC (Cheung et al. [2022](#)). MicroRNAs control cellular processes, including the ovarian and endometrial carcinogenesis, by mediating cell proliferation, immune response or cell death. Indeed, they can function as either oncogenes (oncoMiRs) or tumor suppressors (tsmiRs) depending on the cell type. Therefore, they can influence the tumor characteristics, proliferation, metastatic potential and response to chemotherapy. For these reasons, they are exploited as diagnostic and therapeutic targets. (Kinose et al. [2014](#); Vasilatou et al. [2015](#); Peng and Croce [2016](#); Staicu et al. [2020](#)).

Extracellular urinary microRNAs

MicroRNAs can be secreted to extracellular space through different mechanisms (Sohel [2016](#); Zhao et al. [2019](#)): (1) passive leakage caused by injury and inflammation through apoptosis or necrosis, (2) active secretion of miRNA encapsulated into a membrane-bound vesicles, such as exosomes (50–90 nm) or (3) active release by forming complex with proteins: Argonaut protein 2 (AGO2) (Arroyo et al. [2011](#)) or high density lipoprotein (HDL) (Vickers et al. [2011](#)). These cell-free miRNAs, known as *extracellular or circulating miRNAs*, can be detected in all body fluids, including serum, plasma, urine, saliva, breast milk, seminal, amniotic, pleural, cerebrospinal (Weber et al. [2010](#)) and peritoneal fluid (Roman-Canal et al. [2019](#)).

Urine is a biological fluid consisting of organic and inorganic compounds, salts, cells like leucocytes, kidney and urothelial cells, excreted tumor cells, and the cell-free nucleic acids, such as miRNAs (Oshi et al. [2021](#)). *Urinary microRNAs* originating from primary tumor can be delivered to the bladder from systemic circulation or through kidney cell transport via exosomes (Thomas et al. [2018](#)). Cell-free miRNA found in blood can pass through the glomerular membrane into the urine because of their small size. Only molecules smaller than 6.4 nm in diameter and a molecular weight not greater than 70 kDa can pass through the nephron (Oshi et al. [2021](#)). Exosomes packed with microRNAs can be transferred from blood to recipient renal cells, where they are either reabsorbed back in circulation or end up excreted into urine (Thomas et al. [2018](#)). Also, miRNA-protein complexes with AGO2 have been detected in human urine (Beltrami et al. [2015](#)), while miRNA bound to HDL currently does not seem to play a role in intrarenal transport and excretion to urine (Thomas et al. [2018](#)).

The miRNA form plays a role in stability, with free miRNAs being more susceptible to degradation than the ones packed inside vesicles or associated with proteins (Mlcochova et al. [2015](#)). Encapsulation or association with (lipo)proteins is a protective mechanism that allows circulating miRNAs to be stable and protected from degradation from high RNase activity present in body fluids. For example, mature Ago-bound miRNAs are found to be 3.9-times more stable than mRNAs in the same cell type (with the half-life of 11.4 h for miRNA compared to 2.9 h for mRNA, respectively) (Reichholf et al. [2019](#)). Certain microRNAs have a fairly strong cellular specificity, such as miR-122 for hepatocytes or miR-1 and miR-133 for cardiomyocytes. On the contrary, numerous microRNAs are not always specific to a cell or a tissue, which is why the best approach would be to assay a panel of microRNAs, which could constitute a "signature" characteristic of a given pathology. Depending on their form of transport, vesicular or non-vesicular, the significance of microRNAs as biomarkers will be quite different. For example, the non-vesicular forms may reflect suffering or cell death by necrosis or necroptosis of a particular tissue. The studies have shown that exosomes are mediators of communication allowing microRNAs to act on signaling pathways other than those which synthesized them. The profile of the microRNAs contained in the exosomes is in part specific to the cell which secreted it and is modified according to the physiological or pathological state in which it is found. Thus, a tumor cell will not secrete the same microRNA profile as a normal cell. The assay of microRNAs in urine can therefore reflect the molecular changes that occur in the cancer cells from which they are derived, and thus provide information to aid diagnosis and therapeutic decision-making. Due to stability under extreme conditions in hospital setting, urinary microRNAs are ideal candidates as clinical biomarkers (Mall et al. [2013](#)).

Urine microRNAs as part of liquid biopsy

Liquid biopsy is a minimally invasive technique for detection and analysis of cancer biomarkers from the liquid biological source, such as blood and urine (Alix-Panabières and Pantel [2021](#)). Liquid biopsy can include analysis of biomarkers, including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), mRNA, miRNA and extracellular vesicles (EV or exosomes) (Alix-Panabières and Pantel [2021](#)). Liquid biopsy represents alternative to conventional tissue biopsy, as it can access the tumor information in easy and non-invasive way. Tumor tissue biopsies are invasive, painful, time-consuming and carry a specific risk of complications. This is particularly highlighted in gynecological cancers, as biopsies of the ovarian and endometrial tissues are not easily feasible. Endometrial biopsy is the most common technique to assess the tissue abnormalities, but in some cases is hard to obtain adequate sample for diagnosis (Braun et al. [2016](#)). Moreover, even with current instrumentation and techniques, ovarian biopsies remain invasive, expensive and with the potential risk of the adverse effects on fertility (Kwok and Johnson [2012](#)). Normally, the histopathological assessment of OC is performed following cytoreductive surgery (Ledermann et al. [2013](#)). On contrary, liquid biopsy is not limited by location or size of the tumor tissue. Sampling of blood and urine can be performed at any time point, regardless of the time in the course of the disease, as they are easily available (Alix-Panabières and Pantel [2021](#)). Although liquid biopsy can be performed with several body fluids, primarily blood, urine became interesting source of cancer information (Oshi et al. [2021](#)). Urine miRNA-based liquid biopsy have previously been reported in bladder cancer, for molecular detection and surveillance of the disease (Satyal et al. [2019](#)). In addition, urine miRNAs have been investigated as non-invasive biomarkers in prostate cancer (Juracek et al. [2022](#)). For example, some of the prostate-cancer miRNA species found are miR-222-3p (Juracek et al. [2022](#)) or miRNA-375 (Saxby et al. [2020](#)). Indeed, oncogenic miRNA-375 have been found to enhance proliferation, and is significantly associated with lymph node involvement and

metastases of prostate cancer (Saxby et al. [2020](#)). Therefore, urine liquid biopsy could present cheap and easy diagnostic tool to address gynecological cancers.

Urinary microRNA analysis

As shown in Fig. 2, after urine sample collection from the patient, the first step in urinary miRNA analysis is the processing of the urine sample that will allow for high quality miRNAs yield. Urine as a sample can be collected in different ways which can affect the results of a test. *First morning urine* is collected in the morning after sleeping and fasting. It typically represents the most accurate sample as urine is very concentrated. *Random urine sample* is collected at any time of the day, when urine is diluted, thus can affect the sensitivity of the results. *Full void urine* sample is defined as the entire urine flush from beginning to the end of urinating (Jordaens et al. [2023](#)). *First-void urine* is typically the first part of urine flush collected at any time of the day. *Midstream clean urine* is collected after discarding the first 10 to 50 mL to remove the contaminants. Midstream clean urine is a golden standard for metabolomics and detection urinary tract infections (Jordaens et al. [2023](#)).

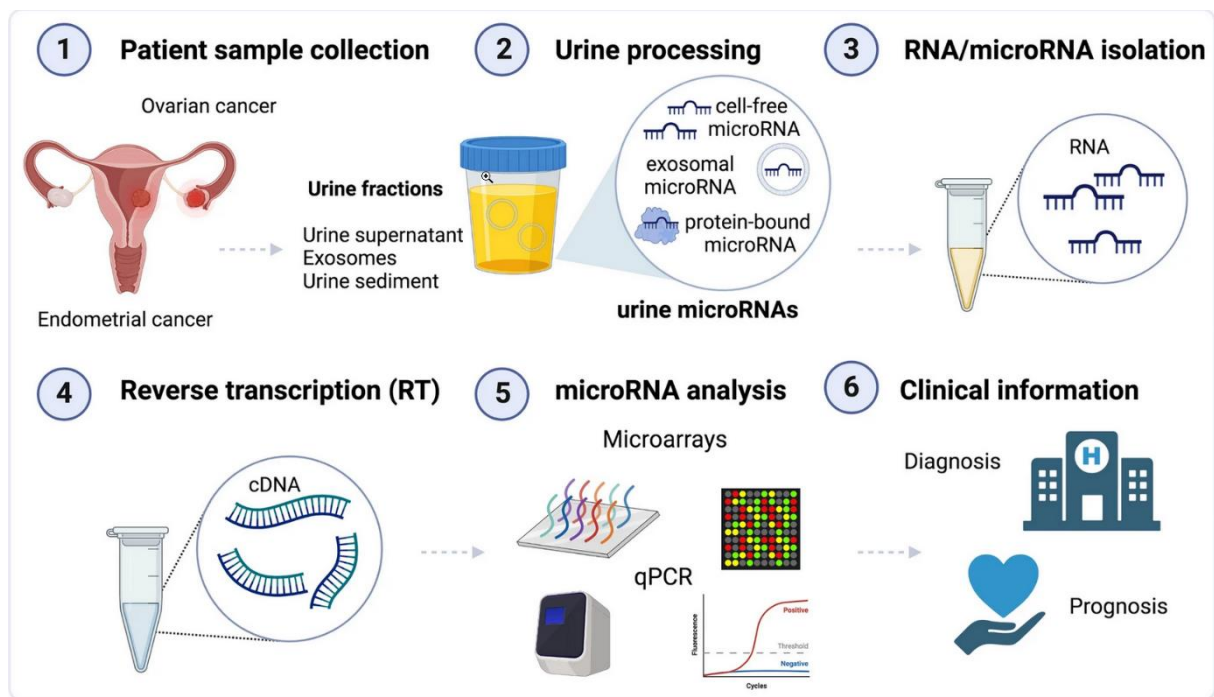


Fig. 2 Overview of the urinary microRNA analysis. After obtaining urine sample from gynecological cancer patient, whole native urine can be processed by centrifugation and separated into different fractions for miRNA analysis : urinary sediment, urine supernatant or exosomal fraction. MiRNAs can be present urine in form of cell-free miRNA, encapsulated miRNA in extracellular vesicles or exosomes, or miRNA coupled with AGO2 proteins. Next, total RNAs or miRNAs are isolated from urine and reverse transcribed (RT) to complementary DNA (cDNA). The analysis is performed by microarrays and/or qPCR in order to identify clinical diagnostic or prognostic cancer-specific miRNA biomarkers. (Created with BioRender.com)

Various studies process the urine samples differently (choice of fraction, transport, storage and centrifugation parameters). However, in most cases, urine samples are collected and stored at -20°C or -80°C until miRNA analysis. The choice of the urine fraction plays an important role in the sample quality. MiRNAs can be detected directly in whole urine or in different *urine fractions* obtained after centrifugation—in the urinary sediment, cell-free urine supernatant or from the exosomes. Typically, cryopreserved native urine specimens are centrifuged to remove

cell debris residues or solid precipitates from pure urine supernatant. Urine sediment is rarely used as it contains not only tumor cells, but cell contamination from urogenital tract. Therefore, pure supernatant is the most common fraction for miRNA analysis. Cell-free urine supernatant can be obtained by centrifugation for 15 min at 3500 rpm at 4 °C. If miRNAs are analyzed in exosomal fraction, the urine sample requires specific processing with commercial kits or ultracentrifugation for extraction of exosomes.

From the selected fraction, miRNAs or total RNA are isolated, reverse transcribed (RT) to complementary DNA (cDNA) and subjected to analysis. The current golden standard for miRNA analysis is quantitative polymerase chain reaction (qPCR), due to its high sensitivity, specificity and ability to be routinely used (Kappel and Keller [2017](#); Ye et al. [2019](#)). Another widely used method for miRNA detection in urine is microarray, as it allows for rapid and high-throughput detection of multiple miRNAs. However, poor sensitivity, long hybridization time and a high price may restrict its clinical application today (Ye et al. [2019](#)).

The urinary miRNA studies can be divided into two phases: (1) discovery phase of screening multiple miRNAs by microarray techniques to find potential candidate miRNA biomarkers and (2) validation of the miRNAs candidates phase by qPCR. Some studies selected for this review choose both phases in order to discover new aberrantly expressed miRNAs. On the other hand, other research groups focus on validation phase, where they choose to analyze specific miRNAs in urine, which were previously reported in tissue or blood of cancer patients.

Urinary microRNAs as biomarkers of gynecological cancers

In the last decade, urine microRNAs have been recognized as useful biomarkers in gynecological cancers. Most studies are performed on patients' samples from endometrial and ovarian cancer, probably due to the fact that effective diagnostic tools are lacking compared to other gynecological cancers, such as cervical cancer, where screening has been widely implemented. Until today, microRNA analysis has not yet been performed or published in other gynecological malignancies, such as cervical, vaginal, etc. Therefore, more research is needed to assess all microRNA signatures in urine across gynecological cancers. The overview of all detected microRNA species and corresponding methodology and clinical data is presented in Table [1](#).

Table 1 Overview of urinary microRNAs as potential biomarkers in breast and gynecological cancers and corresponding methodology in clinical samples

Cancer	microRNA	Urine fraction	Expression pattern in cancer compared to control	Number of cancer patients enrolled in the study	Number of controls enrolled in the study	Method of total RNA or microRNA isolation	Method of miRNA detection/quantification	References
Ovarian	miR-30a-5p	Supernatant Exosomes	Upregulated	OC = 39	BC = 26 HC = 30	mirVana miRNA Isolation kit (Ambion, Inc., Austin, TX, USA)	<i>Profiling miRNAs:</i> miRNA Microarray <i>Candidate miRNAs:</i> RT-qPCR	Zhou et al. (2015)
Ovarian	miR-92a miR-106b	Supernatant	Upregulated Downregulated	OC = 6	HC	Urine Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada)	RT-qPCR	Záveský et al. (2015)
Ovarian	miR-200a miR-200b miR-200c	Supernatant Exosomes	No significant difference	OC = 9	BC = 7	NucleoSpin® miRNA Plasma kit (Macherey–Nagel GmbH, Düren, Germany)	RT-qPCR	Savolainen et al. (2020)
Ovarian	miR-15a let-7a miR-10a	Whole urine	Upregulated Downregulated Downregulated	OC = 13	HC = 17	miRCURY RNA Isolation Kit—Biofluids (Exiqon, Qiagen GmbH, Hilden, Germany)	RT-qPCR	Berner et al. (2022)
Ovarian + Endometrial	miR-10b-5p miR-205-5p	Supernatant	Upregulated (EC + OC) Downregulated (EC)	OC = 10 EC = 10	HC = 30	Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada)	<i>Profiling miRNAs:</i> miRNA Microarray <i>Candidate miRNAs:</i> RT-qPCR	Ritter et al. (2020)
Endometrial	miR-106b	Supernatant	Downregulated	EC = 5 BC = 2	HC = 12	Urine Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada)	RT-qPCR	Záveský et al. (2015)
Endometrial	miR-155-5p, miR425-5p, miR23a-3p miR-200c	Exosomes	Upregulated Upregulated Upregulated Upregulated	EC = 12	HC = 5	miRNA isolation (details not available)	RT-qPCR	Ruskin et al. (2016)
Endometrial	miR-200c	Exosomes	Upregulated	EC = 22	HC = 5	mirVana miRNA isolation kit (Ambion, Inc., Austin, TX, USA)	<i>Profiling miRNAs:</i> Human miFinder miRNA PCR array kit <i>Candidate miRNAs:</i> RT-qPCR	Srivastava et al. (2018)
Endometrial	miR-223	Supernatant	Upregulated	EC = 24	BC = 37	Isolation: Urine Total RNA Purification Kit (Norgen Biotek Corp., Thorold, Canada)	RT-qPCR	Donkers et al. (2021)

OC ovarian cancer, EC endometrial cancer, BC benign control, HC healthy control, RT-qPCR reverse transcription-quantitative real-time polymerase chain reaction

From: [Extracellular urinary microRNAs as non-invasive biomarkers of endometrial and ovarian cancer](#)

Ovarian cancer

In ovarian cancer, several urinary miRNAs have been investigated. In the study by Zhou et al., the microarray data identified different miRNA expression patterns in urine from 39 patients with ovarian serous carcinoma, 26 patients with benign gynecological diseases (ovarian cysts, uterine fibroids and endometriosis) and 30 healthy controls. MiR-30a-5p was significantly upregulated and 37 different miRNAs were downregulated in OC which was confirmed by RT-qPCR in the same study. The upregulation was associated with clinical stage (level of miR-30a-5p was higher in stage I/II than in III/IV) and histological grade (more expressed in well-differentiated than in poorly differentiated cancers). To assess the diagnostic value of miR-30a-5p, the ROC curve demonstrated high specificity and sensitivity, with an area under the ROC curve (AUC) of 0.862, suggesting high discrimination potential between cancer and healthy/benign. Specificity for OC was further investigated by comparison of miR-30a-5p expression in ovarian, gastric and colon carcinoma. Reduced urinary level in other cancers suggests that upregulation of urinary miR-30a-5p level may be specific for ovarian serous carcinoma. In addition, miR-30a-5p levels are affected by the surgical removal of the tumor. Reduced urine levels of miR-30a-5p after surgery further supports the idea that miR-30a-5p originates from cancerous ovarian tissue. Moreover, the knockdown of this miRNA inhibited the proliferation and migration of OC cells in vitro. (Zhou et al. 2015). A pilot study by Záveský et al., with six cancer patients with epithelial (serous and mucinous) ovarian cancer, fallopian tube cancer, mixed ovarian/endometrial cancer and healthy controls, focused on candidate cell-free miRNA biomarkers were examined by RT-qPCR in urine of patients with OC. No

statistically significant differences were found in miRNAs isolated from exosomes. However, in supernatant, miR-92a was significantly up-regulated and miR-106b significantly downregulated in OC compared to control samples. While these two candidates are promising, the small number of patients ($n=6$) and different histotypes of ovarian cancer analyzed (Záveský et al. [2015](#)) are important limitations of the study. MiR-92a was previously found to be involved in cervical cancer cell proliferation, migration and invasion, by direct regulation of PI3KR1 expression, regulation of PI3K/AKT and PTEN pathways (Wang et al. [2021](#)). Moreover, in exosomes isolated from blood, miR-92a was found to be slightly upregulated in epithelial ovarian cancer patients versus healthy individuals (Pan et al. [2018](#)). On the other hand, miR-106b is found upregulated in several cancers (lung, prostate, gastric, liver, esophagus) and is an emerging cancer target (Sagar et al. 2021). Therefore, its reduced expression in OC could be potentially a tool to discriminate from other cancers.

The expression signature of mirRNA200 family (miR200a, miR200b and miR200c) was analyzed in tissue, plasma and urine of OC patients. One of the aims was to test whether urine and plasma miRNA levels can reflect each other, and could urine replace or complement blood samples. Savolainen and colleagues focused on correlation between tissue, urine and plasma miRNA levels of the mirRNA200 family (miR200a, miR200b and miR200c). Among the 16 patients enrolled, nine were high-grade serous OC patients with large tumor and 7 patients had benign ovarian mass. Exosomal fraction was precipitated from urine supernatant after centrifugation. MiRNA was isolated from exosomes, reversed transcribed and RT-qPCR was performed. The expression analysis in all three samples (urine, plasma and tissue) showed that miR200a, miR200b and miR200c had similar expression profiles within each sample. The authors found correlations in relative expression of all three miRNAs between urine and plasma in malignant ovarian cancer, with most patients with high expression in plasma having also high expression in urine. However, there was no significant difference between expression profiles in malignant and benign samples, so using exosomal urinary miR-200 signature could not discriminate between two groups (Savolainen et al. [2020](#)). According to the study by Berner et al., using microRNA signatures of different miRNA species could distinguish between OC and healthy. Expression profile of 12 different microRNAs was analyzed in urine of 13 OC patients (12 serous adenocarcinoma and 1 Sertoli-Leydig-Cell tumor) and 17 healthy controls. The results showed that miR-15a was upregulated and let-7a and miR-10a downregulated in OC when compared to healthy controls, suggesting they can be used as biomarkers of OC (Berner et al. [2022](#)). Indeed, miR-15a was found to act as tumor suppressor, by targeting oncogenes such as BCL2, Cyclin D and WNT3, and is often found downregulated in cancer (Otmani and Lewalle [2021](#)). Therefore, its overexpression could help increase discrimination between OC over other cancers and pathologies. In contrast, miR-10a is classified both as an oncogene and as a tumor suppressor, so its utility would be limited in an OC detection assay (Otmani and Lewalle [2021](#)). However, research in standardized (choice of urine fraction and treatment of urine) studies on bigger cohorts are required to validate the deregulation of listed urinary miRNAs for use as non-invasive biomarkers in clinical setting.

Endometrial cancer

In endometrial cancer, several studies have been analyzing urinary miRNAs. In the study by Záveský et al., using qPCR profiling of the 18 cell-free miRNA candidates from urine supernatant from 5 patients with EC, revealed that only miR-106b was significantly down-regulated in EC when compared to control samples (similar to OC). In that same study, exosomes isolated from urine of the same patients were analyzed, but no significant difference were found between endometrial cancer and control samples (Záveský et al. [2015](#)). Urine-

derived exosomes are getting more attention as a source of biomarkers, including miRNAs. In a study by Ruskin et al., researchers analyzed miRNAs from urinary exosomes in EC patients. The urine was collected preoperatively from 12 women undergoing surgical staging and 5 healthy controls. Exosomes were isolated and miRNA expression was analyzed using RT-qPCR. Urinary exosomes from cancer patients and healthy controls exhibited different miRNA expression profile. Several miRNAs, such as miR-155-5p, miR425-5p, miR23a-3p and miR-200c were found to be relatively upregulated in EC (Ruskin et al. [2016](#)). miR-155-5p is a known oncogene upregulated in human cancer (Pasculli et al. [2020](#)). It is currently studied as a potential predictor of chemotherapy response in breast cancer (Pasculli et al. [2020](#)). Similar findings of miRNA expression were observed by the same team by Srivastava et al. Urine-derived exosomes were used to evaluate miRNA expression pattern in EC. Subjects were 22 patients with EC and 5 patients with symptoms of EC, such as abnormal uterine bleeding, thickening of the endometrium or uterine lesions, but without established diagnosis. After isolation and characterization of exosomes from urine, total RNA was isolated, reverse transcribed and subjected to miRNA profiling. Initially, miRNA PCR array was used for analysis of expression of a panel of most biologically significant miRNAs. 84 mature miRNAs were analyzed simultaneously and miR200c-3p was most enriched in EC expression status of miR200c-3p was further validated by RT-qPCR. Based on these results, miRNA-200c could be a potential biomarker for liquid biopsy screening in EC patients (Srivastava et al. [2018](#)).

Recently, the knowledge of miRNA expression patterns in urine of gynecological cancers was expanded by the study by Ritter et al. The urine samples were collected from 10 patients with EC, 10 patients with OC, and 30 healthy controls (HC). In the discovery phase, miRNA microarray chip analysis in cell-free supernatant of urine revealed novel miRNA candidates that were subsequently validated by RT-qPCR. In the gynecological tumors group (EC + OC), the tendency of miR-10b-5p upregulation was observed. In contrast, miR-205-5p seems to be downregulated when compared to healthy control group, especially in EC (Ritter et al. [2020](#)).

Moreover, the detailed expression pattern covering wide range of microRNA species was evaluated in EC and correlated with clinical parameters, such as obesity and associated comorbidities. Urine samples were collected from women presenting with abnormal uterine bleeding, including 24 EC and 37 benign conditions. Urinary miRNAs were isolated and quantified from pure urine supernatant using RT-qPCR. The higher expression levels of miR-223 were observed in urine of women with EC. Furthermore, urinary microRNAs were significantly different in obese women (let7-i, miR-34a, miR-200c, miR-424), in elderly (miR-148a, miR-222), and in women with multiple comorbidities (miR-16, miR-26b, miR-200c). Therefore, miRNAs from urine can correlate with clinical characteristics and miR-223 is potential candidate biomarker for EC (Donkers et al. [2021](#)).

Advantages and challenges of microRNA utilization as ovarian and endometrial cancer biomarkers

Advantages of microRNAs

Endometrial and ovarian cancer are characterized by high mortality rate with late diagnosis remaining a key factor for the poor prognosis due to the lack of screening or early-stage diagnostic methods. Usually, blood is the primary sample for biomarker discovery due to its well-known cellular and proteomic constitution and physiological functions. However, urine is becoming an interesting source for biomarkers owing to certain advantages. First of all, as there is no homeostasis mechanism in urine, excreted products levels and molecular architecture stay stable, are often intact, and they reliably reflect in vivo pathophysiological state. Urine can also

accommodate more changes in early stage of disease, so urinary analysis could lead to earlier diagnosis and treatment intervention (Harpole et al. [2016](#); Jing and Gao [2018](#)). In addition, urine is an easy to access sample, with a high volume availability, that is generally well accepted by patients (Schaafsma et al. [2022](#)). In fact, it was found that gynecological examinations can cause fear and anxiety among women and could cause delay in visiting doctor's office (O'Laughlin et al. [2021](#)). Therefore, non-invasive urine sampling could improve women enrollment in diagnostic procedures and reduce the time for getting the diagnosis. From a technical perspective, urine collection is cheaper and safer than blood sampling. Moreover, since the urinary proteome is less complex, interferences of the sample matrix are reduced during RNA extraction and subsequent analysis (Grayson et al. [2019](#)).

Recently, microRNAs have emerged as potential biomarkers because of their specific characteristics: aberrant expression in cancer, ubiquity in body fluids, and stability in clinical samples. More specifically, urinary miRNAs are stable under clinically relevant temperatures and storage conditions of urine samples (Mall et al. [2013](#)). The expression level of urinary miRNAs stay unchanged under different temperatures and freeze–thaw cycles, which makes them suitable for long-term storage and analysis in clinical environment (Mall et al. [2013](#)). Even under the extreme conditions such as high RNases activity or low or high pH, such as in urine, miRNAs maintain their structural features and resist destruction (Mlcochova et al. [2015](#)). Therefore, miRNA stability in urine further suggest use of urine as a relevant source of miRNA biomarkers. Recent usage of urine miRNAs as diagnostic tools in kidney (Frantzi et al. [2014](#); Di Meo et al. [2020](#)), bladder (Hanke et al. [2010](#); Zhang et al. [2014](#)) and prostate cancer (Catto et al. [2011](#); Fredsøe et al. [2018](#)) provides the insight miRNA utility in gynecological cancers.

Dysregulation of miRNAs and the role in pathophysiology of both EC and OC is well known (Vasilatou et al. [2015](#); Ghafouri-Fard et al. [2020](#)). Numerous studies have focused on expression profiles in tumor tissue and extracellular miRNAs in as biomarkers of EC and OC and are extensively reviewed elsewhere (Yanokura et al. [2010](#); Kinose et al. [2014](#); Delangle et al. [2019](#); Ghafouri-Fard et al. [2020](#); Staicu et al. [2020](#)). In this review, we focused on urinary miRNAs with a question: “could urine complement or even replace blood analysis, while being less invasive?” Based on the current knowledge, the most promising urinary microRNA candidates are miR30a-5p for OC and miR200 family for both EC and OC. miR30a-5p is a potential biomarker of OC, found upregulated both in urine and tissue (Zhou et al. [2015](#)), as well as in blood (Ayaz et al. [2014](#)) and ascites of OC patients (Záveský et al. [2019](#)). The miR30a-5p function as a mediator in carcinogenesis (Wang et al. [2018](#)), migration (Zhou et al. [2015](#)) and metastatic potential of OC cells (Wang et al. [2019](#)). This may imply that miR30a-5p could be both a diagnostic and a therapeutic target for OC, specifically high-grade serous subtype.

Mir200 family (miR200a, miR200b, miR200c, miR-141 and miR-429) and are highly expressed miRNAs in OC and EC (Koutsaki et al. [2017](#)). They have been previously detected in EC an OC tissue and blood as well. Levels of miR200 family members were correlated with different clinicopathological features, such as prognosis and disease survival (Cao et al. [2014](#); Vilming Elgaaen et al. [2014](#); Zuberi et al. [2015](#); Wilczynski et al. [2018](#)). MiRNAs200 family were detected in exosomes from urine in both OC (Savolainen et al. [2020](#)) and EC (Srivastava et al. [2018](#)). However, more studies are needed to evaluate the clinical utility of urinary miR200s family as a biomarker for gynecological malignancies.

Limitations of urine microRNAs

Although miRNAs do have potential, no standardized workflows or recommendations have not been established for measurement of miRNAs in body fluids (Kappel and Keller [2017](#)). When interpreting results of urinary miRNA expression, several physiological and methodological limitations have to be considered. Indeed, a higher level of inconsistent patterns and higher variations in urine miRNAs were observed than when analyzing samples from tissue, plasma from OC patients. (Savolainen et al. [2020](#)). This could be partially explained by diversity of subtypes, stage of the disease and treatments in selected patients. Furthermore, what is observed is heterogeneity of results of same miRNA in urine when compared to tissue or blood. Until now, there is still not enough evidence about the correlation of miRNA levels between tissue, blood and urine nor the metabolic pathways that could affect the miRNA levels in each sample. The regulatory mechanisms about selective secretion from tumor cells into extracellular environment or secretion to urine are still unknown. There is a possibility tumor cells preferentially release or retain specific miRNA subpopulations. The question is could changes in tissue miRNA expression be reflected in serum or urinary levels and what is the relationship between different body compartments.

Moreover, the studies selected in this review are using different approaches and methodologies. To be able to implement miRNAs as biomarkers in gynecological oncology, standardization of miRNA research is required. More specifically, there is a need in consensus about (1) urine preparation and defined choice of urine fraction because enrichment can vary, as observed in the study by Závěský et al. where results for the same miRNA were different between supernatant and exosomes (2) methods of RNA or exosome isolation and miRNA detection from urine; and (3) normalization of data in qPCR results. Different normalization procedures are used for extracellular miRNA measurement because there are no standardized genes or miRNA candidates for normalization purposes (Kappel and Keller [2017](#)).

Overall, the above studies provided valuable urine biomarker information for EC and OC. Based on the current findings, a single individual urinary miRNA biomarker is not adequate for diagnosis of gynecological cancer. Multiparametric approach could improve the performance of miRNAs as biomarkers knowing that urinary miRNA “signatures” could discriminate between both EC and OC and healthy. Detection of the deregulated miRNAs in urine in conjunction with symptoms could be potentially used as preliminary diagnostic method which can be further substantiated with clinical examination. Therefore, miRNAs could be useful as diagnostic tool in future development of early phase and screening methods. MiRNAs from urine are particularly interesting targets for laboratory applications because they can be collected in an easy, non-invasive way. Moreover, qPCR detection method is already available in routine clinical laboratories. Although miRNA-based diagnostic tests have not paved the way into the clinical routine yet, there is several promising miRNA candidates, such as miR200 family, which may eventually lead to routine applications in future. More standardized multi-institutional studies on big cohorts are needed for implementation of urinary miRNAs as diagnostic biomarkers in gynecological oncology.

Conclusion

Urine microRNA have been shown to be useful non-invasive diagnostic biomarkers in gynecological oncology, in both ovarian and endometrial cancer patients. Urine microRNA signatures could successfully differentiate and stratify cancer patients from healthy individuals or individuals with benign gynecological conditions. However, in order to minimize the variability of results, it is essential to standardize processing of the urine samples, choice of detection methods and microRNA analysis strategies. In addition, combination of several

microRNAs through "signatures" presents an alternative to the lack of tumor specificity of an individual microRNA species. Although miRNA-based diagnostic tests have not found their way into the clinical routine yet, there is several promising microRNA candidates that may eventually lead to routine applications in future. Aberrant expression of miR200 family has been especially highlighted, suggesting their potential role as a liquid biopsy biomarkers. More multi-institutional studies on big cohorts are needed before implementation of urinary miRNAs as diagnostic biomarkers in gynecological oncology.

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Contributions

AH and A-CED had the idea for the manuscript. The first draft of the manuscript was written by AH. A-CED and CS critically revised the work. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Ethics declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent and ethical approval

Not required for this type of study.